

# Pyrenophoric Acids B and C, Two New Phytotoxic Sesquiterpenoids Produced by *Pyrenophora semeniperda*

Marco Masi,<sup>†,‡</sup> Susan Meyer,<sup>§</sup> Alessio Cimmino,<sup>‡</sup> Suzette Clement,<sup>§</sup> Beth Black,<sup>†</sup> and Antonio Evidente<sup>\*,‡</sup>

<sup>†</sup>Department of Plant and Wildlife Sciences, Brigham Young University, Provo, Utah 84601, United States

<sup>‡</sup>Dipartimento di Scienze Chimiche, Università di Napoli Federico II, Complesso Universitario Monte Sant' Angelo, Via Cintia 4, 80126 Napoli, Italy

<sup>§</sup>Shrub Sciences Laboratory, US Forest Service Rocky Mountain Research Station, 735 North 500 East, Provo, Utah 84606, United States

## S Supporting Information

**ABSTRACT:** Two new phytotoxic sesquiterpenoid acids, named pyrenophoric acids B and C, were isolated together with the related pyrenophoric and abscisic acids from solid *Bromus tectorum* (cheatgrass) seed culture of the seed pathogen *Pyrenophora semeniperda*. This fungus has been proposed as a mycoherbicide for biocontrol of cheatgrass (*Bromus tectorum*), a Eurasian annual grass that has become invasive in rangelands and is also a serious agricultural weed in the western U.S. Pyrenophoric acids B and C were characterized by spectroscopic methods (NMR and HR ESIMS) as (2Z,4E)-5-[(1R\*,4R\*,6R\*)-1,4-dihydroxy-2,2,6-trimethylcyclohexyl]-3-methylpenta-2,4-dienoic and (2Z,4E)-5-[(1S\*,3S\*,4R\*,6S\*)-3,4-dihydroxy-2,2,6-trimethylcyclohexyl]-3-methylpenta-2,4-dienoic acids, respectively. Cytochalasins A, B, F, and Z3, as well as deoxaphomin and pyrenophoric acid, all previously isolated from *P. semeniperda* grown on wheat seed, were also isolated from cheatgrass seed culture. In a cheatgrass seedling bioassay at 10<sup>-3</sup> M, pyrenophoric acid B showed higher coleoptile toxicity than pyrenophoric acid, while pyrenophoric acid C showed lower phytotoxicity. Abscisic acid was by far the most active compound.

**KEYWORDS:** *Bromus tectorum*, *Pyrenophora semeniperda*, sesquiterpenoids, pyrenophoric acids B and C, cytochalasins, mode of action

## INTRODUCTION

*Bromus tectorum* L. (cheatgrass, downy brome) is an exotic winter annual grass weed that causes serious losses in intensive agriculture, particularly in winter cereal crops,<sup>1,2</sup> and is also a major problem on rangelands in the western U.S.<sup>3</sup> The fungus *Pyrenophora semeniperda* (Brittlebank and Adams) Shoemaker, a naturally occurring necrotrophic seed pathogen, has been proposed as a potential biocontrol agent for this weed.<sup>4</sup> *Pyrenophora semeniperda* is a generalist pathogen with a cosmopolitan distribution.<sup>5,6</sup> Its ability to produce toxins in vitro, which could potentially be useful in biological control strategies, was first investigated by Evidente et al., 2002.<sup>7</sup> The study of an Australian strain revealed that the fungus produced a large amount of the phytotoxic cytochalasin B, as well as cytochalasin A, F, deoxaphomin, and the three novel cytochalasins, Z1, Z2, and Z3, when grown on wheat seeds. The production of all of these compounds except cytochalasins Z1 and Z2 was confirmed working with solid wheat seed cultures of ten strains collected from Utah (USA) populations, and a rapid and sensitive HPLC method for quantification of cytochalasin B in the organic extracts was developed.<sup>8</sup> Recently a new phytotoxic sesquiterpenoid characterized as (2Z,4E)-5-[(1S,3S,4R,6S)-1,4-dihydroxy-2,2,6-trimethylcyclohexyl]-3-methylpenta-2,4-dienoic acid, named pyrenophoric acid and closely related to abscisic acid, was isolated from wheat seed culture<sup>9</sup> of one of the strains most active in a *B. tectorum* coleoptile bioassay.<sup>8</sup> The fungus was also grown in potato dextrose broth culture, and a new spirocyclic  $\gamma$ -lactam, named

spirostaphylotrichin W, was isolated together with the well-known and closely related spirostaphylotrichins A, C, D, R, and V, as well as triticone E. The structure and biological characterization of the new spirostaphylotrichin W have been described.<sup>10</sup>

This manuscript reports the chemical and biological characterization of two new phytotoxic metabolites isolated from *P. semeniperda* together with some already known compounds and discusses their possible role in pathogenesis on *B. tectorum* seeds.

## MATERIALS AND METHODS

**General Experimental Procedures.** Optical rotations were measured in CHCl<sub>3</sub> unless otherwise noted, on a PerkinElmer 241 polarimeter (Norwalk, CT, USA); IR spectra were recorded as glassy film on a Thermo Nicolet Avatar 370 FT-IR spectrometer (Madison, WI, USA); UV spectra were recorded in MeOH solution on a Hewlett-Packard 8453 UV/vis spectrophotometer (Palo Alto, CA, USA); <sup>1</sup>H and <sup>13</sup>C NMR spectra were recorded in CDCl<sub>3</sub> at 500 MHz on a Varian instrument (Palo Alto, CA, USA). The same solvent was used as internal standard. Carbon multiplicities were determined by distortionless enhancement by polarization transfer (DEPT) spectra.<sup>11</sup> DEPT, correlation spectroscopy (COSY)-45, heteronuclear single quantum coherence (HSQC), heteronuclear multiple-bond correlation

Received: July 24, 2014

Revised: September 24, 2014

Accepted: September 28, 2014

Published: September 28, 2014

Table 1.  $^1\text{H}$  and  $^{13}\text{C}$  NMR Data of Pyrenophoric Acids B and C (1 and 2)<sup>a</sup>

position	1			2		
	$\delta_{\text{C}}^b$	$\delta_{\text{H}}$ (J in Hz)	HMBC	$\delta_{\text{C}}$	$\delta_{\text{H}}$ (J in Hz)	HMBC
1	169.1 C			169.0 C		
2	116.0 CH	5.72 (1H) s <sup>c</sup>	Me-6	114.9 CH	5.58 (1H) s <sup>c</sup>	Me-6
3	152.1 C		Me-6	153.2 C		Me-6
4	128.7 CH	7.78 (1H) d (15.9)	H-2, H-12, Me-6	132.0 CH	7.55 (1H) d (15.7)	Me-6
5	138.0 CH	6.32 (1H) d (15.9)		136.7 CH	6.34 (1H) dd (15.7, 9.9)	
6	21.6 CH <sub>3</sub>	2.04 (3H) s <sup>c</sup>		21.4 CH <sub>3</sub>	2.06 (3H) s <sup>c,d</sup>	
7	79.6 C		H-5, Me-13, Me-14	53.4 CH	2.20 (1H) dd (9.9, 5.1)	Me-15
8	38.7 C		Me-14, Me-15	38.9 C		Me-15
9	44.7 CH <sub>2</sub>	1.73 (2H) m		69.2 CH	4.38 (1H) m	
10	66.9 CH	4.13 (1H) quint (3.2)		69.0 CH	3.77 (1H) quint (4.4)	
11	30.4 CH <sub>2</sub>	1.76 (1H) br d (15.1) 1.51 (1H) ddd (15.1, 13.0, 3.2)		38.5 CH <sub>2</sub>	1.91 (1H) dt (12.7, 4.4) 1.71 (1H) quint (7.0)	
12	20.6 CH	2.36 (1H) m		21.4 CH	2.06 (1H) m <sup>d</sup>	
13	25.1 CH <sub>3</sub>	1.27 (3H) s		29.4 CH <sub>3</sub>	1.25 (3H) s <sup>d</sup>	H-7
14	29.1 CH <sub>3</sub>	1.25 (3H) s		29.4 CH <sub>3</sub>	1.25 (3H) s <sup>d</sup>	H-7
15	15.6 CH <sub>3</sub>	0.84 (3H) d (6.8)		18.5 CH <sub>3</sub>	1.05 d (3H) (7.6)	H-12

<sup>a</sup>The chemical shifts are in  $\delta$  values (ppm) from TMS. 2D  $^1\text{H}$ ,  $^1\text{H}$  (COSY) and 2D  $^{13}\text{C}$ ,  $^1\text{H}$  (HSQC) NMR experiments delineated the correlations of all protons and the corresponding carbons. <sup>b</sup>Multiplicities were assigned by DEPT spectra. <sup>c</sup>These signals are allylic coupled. <sup>d</sup>These signals are overlapped.

(HMBC), and nuclear Overhauser enhancement spectroscopy (NOESY) experiments<sup>11</sup> were performed using Varian microprograms. HRESI MS were recorded on an Agilent Technologies 6120 quadrupole LC/MS (Santa Clara, CA, USA) instrument. Analytical and preparative TLC were performed on silica gel (Kieselgel 60, F<sub>254</sub> plates, 0.25 and 0.5 mm) (Merck, Darmstadt, Germany). The spots were visualized by exposure to UV radiation (254 nm) and/or by spraying first with 10% H<sub>2</sub>SO<sub>4</sub> in MeOH and then with 5% phosphomolybdic acid in EtOH, followed by heating at 110 °C for 10 min. Column chromatography was performed on a silica gel column (Kieselgel 60, 0.063–0.200 mm) (Merck).

**Fungal Strain.** The *P. semeniperda* strain WRK10-22 used in this study was obtained from a *B. tectorum* seed bank sample collected from the Whiterocks enclosure (−112.7780 longitude 40.3282 latitude, 1446 m elevation) in Skull Valley, Tooele County, Utah, USA, in November 2010. For details see Masi et al.<sup>8</sup>

**Production, Extraction, and Purification of Phytotoxins.** To produce solid culture on *B. tectorum* seeds, 6.6 mg of WRK10-22 conidia suspended in sterile H<sub>2</sub>O were added to 200 g of soaked, autoclaved *B. tectorum* seeds, and the mixture was placed in a sterile 1 L Erlenmeyer flask with an aluminum foil cap at 22 °C. The flask was hand-shaken periodically during the 4-week incubation period to prevent caking together of the grains. The culture was then spread in pans and air-dried for at least several weeks prior to extraction. The dried material was then minced using a laboratory mill and extracted with 500 mL of MeOH/H<sub>2</sub>O (1% NaCl) (55:45, v/v). The mixture was centrifuged for 1 h at 10,000 rpm. The pellet was extracted again with the same solvent mixture in the same conditions, and the two supernatants were then pooled (pH 5.5), defatted by *n*-hexane (2 × 500 mL), and extracted with CH<sub>2</sub>Cl<sub>2</sub> (3 × 500 mL). The resulting aqueous phase was acidified to pH 2 with formic acid and extracted with EtOAc (3 × 500 mL).

The EtOAc organic extracts were combined, dried (Na<sub>2</sub>SO<sub>4</sub>), and evaporated under reduced pressure to yield a brown solid residue (193.4 mg) that showed significant phytotoxic activity in a *B. tectorum* coleoptile bioassay. It was then subjected to bioassay-guided fractionation through column chromatography (750 mm × 30 mm) on silica gel, eluted with 1 L of EtOAc/MeOH/H<sub>2</sub>O (75:20:5, v/v/v), affording six groups of homogeneous fractions. The residue (16.4 mg) of the second fraction was further purified by TLC on silica gel, eluted with CH<sub>2</sub>Cl<sub>2</sub>/MeOH (9:1, v/v), yielding pyrenophoric acid, 3 (*R*<sub>f</sub> 0.36, 10.2 mg), as a homogeneous amorphous solid. The residue (14.6 mg) of the third fraction was further purified by TLC on silica gel,

eluted with CH<sub>2</sub>Cl<sub>2</sub>/MeOH (9:1, v/v), yielding abscisic acid, 4 (*R*<sub>f</sub> 0.30, 3.2 mg, 16 mg/kg), as a white crystal powder and a mixture of two compounds. The mixture was further purified by TLC on silica gel, eluted with EtOAc/MeOH/H<sub>2</sub>O (9.5:0.3:0.2, v/v/v), yielding pyrenophoric acid B, 1 (*R*<sub>f</sub> 0.62, 1.8 mg, 9 mg/kg), and pyrenophoric acid C, 2 (*R*<sub>f</sub> 0.72, 1.6 mg, 8 mg/kg), both as homogeneous amorphous solids.

The CH<sub>2</sub>Cl<sub>2</sub> organic extracts were combined, dried (Na<sub>2</sub>SO<sub>4</sub>), and evaporated under reduced pressure to yield a brown solid residue (694.4 mg), which essentially contained cytochalasin B (7) as shown by TLC analysis carried out in comparison with an authentic sample of the toxin. The mixture was washed with small aliquots (5 × 1 mL) of MeOH, and 7 was crystallized twice from EtOAc-*n*-hexane (1:5, v/v), giving white needles (223.2 mg). The mother liquors of cytochalasin B crystallization (155.3 mg) were combined with the initial MeOH washing (for a residual total extract of 470.1 mg) and fractionated by different steps of column chromatography and TLC on silica gel, affording a further amount of 7 (118.6 mg, for a total of 341.8 mg, 1025.4 mg/kg) and pyrenophoric acid (3, 6.3 mg, for a total of 16.5 mg, 82.5 mg/kg) as well as cytochalasin A (5, 12.3 mg, 61.5 mg/kg), cytochalasin F (9, 7.9 mg, 23.7 mg/kg), cytochalasin Z3 (8, 0.8 mg, 4.0 mg/kg), and deoxaphomin (6, 18.3 mg, 91.5 mg/kg).

**Pyrenophoric Acid B (1).** [ $\alpha$ ]<sub>D</sub><sup>25</sup>: +21.3 (*c* = 0.18); IR  $\nu_{\text{max}}$  3394, 1727, 1670, 1554, 1260 cm<sup>−1</sup>; UV  $\lambda_{\text{max}}$  nm (log  $\epsilon$ ) 256 (4.75);  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra, see Table 1; HRESI MS (+) spectrum *m/z* 286.1979 [C<sub>15</sub>H<sub>28</sub>NO<sub>4</sub>, calcd 286.2018, M + NH<sub>4</sub>]<sup>+</sup>; HRESI MS (−) spectrum *m/z* 267.5917 [C<sub>15</sub>H<sub>23</sub>O<sub>4</sub>, calcd 267.1596, M − H]<sup>−</sup>.

**Pyrenophoric Acid C (2).** [ $\alpha$ ]<sub>D</sub><sup>25</sup>: +67.7 (*c* = 0.16); IR  $\nu_{\text{max}}$  3412, 1731, 1668, 1556, 1260 cm<sup>−1</sup>; UV  $\lambda_{\text{max}}$  nm (log  $\epsilon$ ) 257 (4.68);  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra, see Table 1; HRESI MS (+) spectrum *m/z* 286.2026 [C<sub>15</sub>H<sub>28</sub>NO<sub>4</sub>, calcd 286.2018, M + NH<sub>4</sub>]<sup>+</sup>; HRESI MS (−) spectrum *m/z* 267.6075 [C<sub>15</sub>H<sub>23</sub>O<sub>4</sub>, calcd 267.1596, M − H]<sup>−</sup>.

**Pyrenophoric Acid (3).**  $^1\text{H}$  NMR spectrum was very similar to that previously reported.<sup>9</sup> HRESI MS (+) spectrum *m/z* 286.2011 [C<sub>15</sub>H<sub>28</sub>NO<sub>4</sub>, calcd 286.2018, M + NH<sub>4</sub>]<sup>+</sup>.

**(+)-Abscisic Acid (4).** [ $\alpha$ ]<sub>D</sub><sup>25</sup>: +270.2 (*c* = 0.22 MeOH) [lit.<sup>12</sup> [ $\alpha$ ]<sub>D</sub><sup>23</sup>: +278.3 (*c* = 0.21, MeOH)]; IR  $\nu_{\text{max}}$  3378, 1648, 1625, 1601, 1261 cm<sup>−1</sup> [lit.<sup>13</sup>  $\nu_{\text{max}}$  (solid) 3394, 1646, 1622, 1597, 1248 cm<sup>−1</sup>];  $^1\text{H}$  NMR spectrum was very similar to that previously reported;<sup>13</sup> HRESI MS (+) spectrum *m/z* 265.1495 [C<sub>15</sub>H<sub>21</sub>O<sub>4</sub>, calcd 265.1505, M + H]<sup>+</sup>.

**Cheatgrass Seedling Bioassay.** Pyrenophoric acids B, 1, and C, 2, pyrenophoric acid, 3, abscisic acid, 4, cytochalasin A, 5, deoxaphomin, 6, cytochalasin B, 7, and cytochalasin F, 9, were first

dissolved in DMSO, and then brought up to the assay concentration of  $10^{-3}$  M or  $10^{-4}$  M with distilled water (the final content of DMSO was 2%). Pyrenophoric acid, 3, and cytochalasins 5, 6, 7, and 9 were also assayed in mixture (1:1 molar ratio) at  $10^{-4}$  M with the same procedure. For each sample and concentration, 1.33 mL of the solution was pipetted into each of three 6 cm Petri dishes onto the surface of one filter paper. Seeds were incubated in 2% DMSO in the control treatment.

Six host seeds were arranged onto the surface of each filter paper in a pattern that made it possible to track individual seeds. Petri dishes were sealed with Parafilm to retard moisture loss and incubated at 20 °C with a 12/12 light/dark photoperiod. Germination was scored each day, and germination day was tracked individually for each seed. Five days after germination, the coleoptile and radicle length of each seedling was measured and recorded using electronic calipers. Most seeds germinated within 3 days. Seeds that did not germinate within 7 days (<5%) were excluded from analysis, while seeds that produced a radicle but no coleoptile were scored with a coleoptile length of zero. Cytochalasin Z3, 8, was not tested because of the small amount obtained.

**Statistical Analysis.** Three separate experiments were performed, each with its own control. The first included the four cytochalasins, 5, 6, 7, and 9, at two concentrations ( $10^{-3}$  and  $10^{-4}$  M), while the second included the three pyrenophoric acids, 1, 2, and 3, and abscisic acid, 4, at the same two concentrations. The third experiment included each additive pairwise combination of cytochalasins 5, 6, 7, and 9 (6 pairs) and pairwise combinations of each cytochalasin with pyrenophoric acid, 3, at  $10^{-4}$  M (4 pairs). Pyrenophoric acids B, 1, and C, 2, could not be included in this last experiment because of the small amounts obtained. The objective of the pairwise tests was to determine whether the combined effects of the different toxins were additive, less than additive, or synergistic.

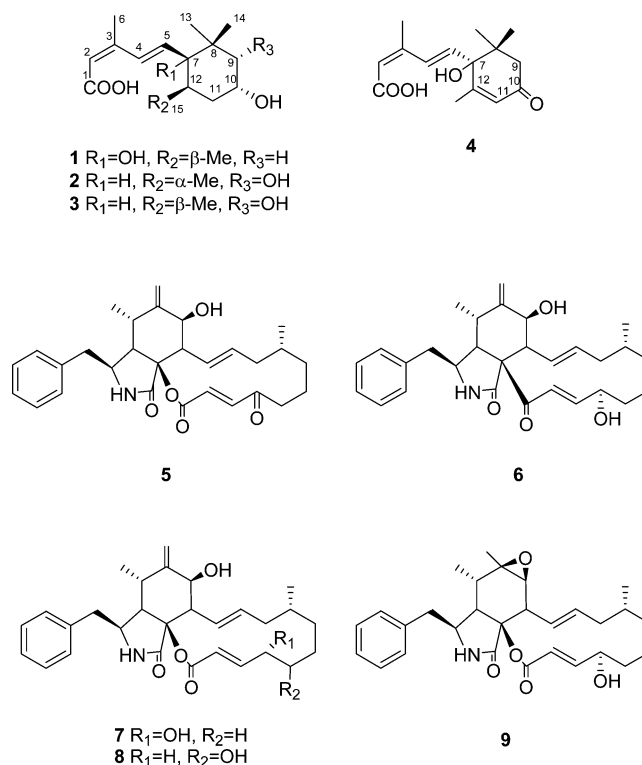
Data from the first two experiments were analyzed using two-way analysis of variance (ANOVA; SAS Proc GLM) with compound and concentration as fixed effects and day-5 coleoptile length, day-5 radicle length, and germination time as the response variables. Data from the third experiment were combined with data from the pure compound bioassays at  $10^{-4}$  M to permit comparisons between pure compounds and pairs of compounds and were then subjected to a similar analysis. LSMeans comparisons from the ANOVAs were used to test the statistical significance of differences between treatment means ( $P < 0.05$ ). Coleoptile and radicle length data were log-transformed to improve homogeneity of variance prior to analysis. Germination percentage was also included in the data presentation but was not analyzed statistically because of inadequate replication.

Coleoptile and radicle length data are presented as reductions relative to the control, i.e.,  $1 - (\text{treatment/control})$ , while germination time data are presented as germination delay, i.e., treatment germination time – control germination time, in order to make the data comparable across experiments with different control treatments. A separate test confirmed that the DMSO controls did not differ significantly across experiments for any response variable.

## RESULTS AND DISCUSSION

When a *P. semeniperda* strain (WRK10-22) was cultured for the first time on host cheatgrass seeds (*B. tectorum*) and the culture was extracted, pyrenophoric acid, 3 (Figure 1), and a large quantity of cytochalasin B (7, Figure 1) were isolated from the  $\text{CH}_2\text{Cl}_2$  extract, together with cytochalasin A, 5, deoxaphomin, 6, cytochalasin Z3, 8, and cytochalasin F, 9 (Figure 1). The physical and spectroscopic properties of these compounds were identical to those previously reported,<sup>7,9</sup> confirming that the fungus is able to produce these metabolites when grown on host seeds (*B. tectorum*).<sup>8,9</sup>

The aqueous phase remaining after  $\text{CH}_2\text{Cl}_2$  extraction was acidified (pH 2) and extracted with EtOAc. From the purification of this organic extract two additional polar metabolites were isolated together with the known abscisic



**Figure 1.** Structures of pyrenophoric acid B, 1, pyrenophoric acid C, 2, pyrenophoric acid, 3, abscisic acid, 4, cytochalasin A, 5, deoxaphomin, 6, cytochalasin B, 7, cytochalasin Z3, 8, and cytochalasin F, 9.

acid, 4 (Figure 1), and a further amount of pyrenophoric acid, 3 (Figure 1). Both polar metabolites showed a molecular formula of  $\text{C}_{15}\text{H}_{24}\text{O}_4$  as deduced from their HRESI MS, consistent with four hydrogen deficiencies. The preliminary investigation of their  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra, carried out in comparison with those of pyrenophoric acid, 3,<sup>9</sup> showed that they are very closely related to 3, and, being new, they were named pyrenophoric acids B, 1, and C, 2 (Figure 1).

The  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra of 1 and 2 showed the signal pattern of a 3-methylpenta-2,4-dienoic acid, linked at C-7 with the same stereochemistry as in 3, also confirmed on the basis of the couplings observed in their COSY, HSQC, and HMBC spectra.<sup>11</sup> However, they differed significantly for the cyclohexyl moiety system.

The  $^1\text{H}$  NMR of 1 (Table 1) differed from that of 3 essentially by the absence of the double doublet of H-7 while H-5 was a doublet ( $J = 15.9$  Hz) at  $\delta$  6.32 instead of the expected double doublet as in 3. H-5 in the COSY spectrum<sup>11</sup> was *trans* coupled with H-4, which showed a doublet ( $J = 15.9$  Hz) at  $\delta$  7.78. As expected, the COSY spectrum also showed the absence of the coupling between H-7 with H-12, which appeared as a multiplet at  $\delta$  2.36. These results and the molecular formula suggested the presence of a tertiary hydroxy group bonded at C-7. This was confirmed by the corresponding signal observed in the  $^{13}\text{C}$  NMR spectrum (Table 1) at the typical chemical shift value of  $\delta$  79.6.<sup>14</sup> This carbon (C-7) significantly coupled in the HMBC spectrum with H-5 and both Me-13 and Me-14. A further significant difference, observed comparing the  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra of 1 to those of 3, was the absence of the signals due to the hydroxylated secondary carbon C-9 and the presence of the signals of a further methylene group ( $\text{H}_2\text{C-9}$ ) at  $\delta$  1.73 (2H)



m/44.7 CH<sub>2</sub>.<sup>14,15</sup> H<sub>2</sub>-9, in the COSY spectrum, coupled with the proton of the adjacent secondary hydroxylated carbon (CH-10), which resonated as a quintet ( $J = 3.2$  Hz) at  $\delta$  4.13, being also coupled with both protons of the other adjacent methylene group (H<sub>2</sub>C-11) appearing as a broad doublet ( $J = 15.1$ ) and a doublet of doublets ( $J = 15.1, 13.0$ , and  $3.2$  Hz) at  $\delta$  1.76 and 1.51. The latter protons (H<sub>2</sub>-11) were also coupled with the multiplet of H-12, and this was coupled in turn with the geminal methyl group (Me-15) appearing as a doublet ( $J = 6.8$  Hz) at  $\delta$  0.84. The other two methyl groups resonated as singlets at  $\delta$  1.27 and 1.25 (Me-13 and Me-14). The couplings observed in the HSQC spectrum allowed us to assign the chemical shifts at  $\delta$  138.0, 128.7, 116.0, 66.9, 30.4, 29.1, 25.1, 20.6, and 15.6 to C-5, C-4, C-2, C-10, C-11, C-14, C-13, C-12 and C-15. Those observed in the HMBC spectrum between C-3 with Me-6 and C-8 with Me-13 and Me-15 allowed us to attribute to these two quaternary carbons the chemical shifts of  $\delta$  152.1 and 38.7. The remaining signal at the typical chemical shift value for a carboxylic group at  $\delta$  169.1 was assigned to C-1.<sup>14</sup> Thus, the chemical shifts were assigned to all the protons and corresponding carbons as reported in Table 1, and **1** could be formulated as 5-(1,4-dihydroxy-2,2,6-trimethylcyclohexyl)-3-methylpenta-2,4-dienoic acid.

This structure was confirmed by the other couplings observed in the HMBC spectrum (Table 1) and by the HRESI MS data, which showed the ammonium cluster  $[M + NH_4]^+$  at  $m/z$  286.1979. When the same spectrum was recorded in negative ion mode, the pseudomolecular ion  $[M - H]^-$  was recorded at  $m/z$  267.5917.

The relative stereochemistry of **1**, as depicted in Figure 1, was deduced from the coupling constants measured in the <sup>1</sup>H NMR spectrum. In particular, also by comparison with the value observed in **3**, the values measured for the coupling constants between H-12 and both protons of H<sub>2</sub>-11 and those for the coupling of H-10 and H<sub>2</sub>-11 and H-9 allowed us to locate H-10 and H-12 as  $\beta$ -equatorial and  $\alpha$ -axial as in **3**. The comparison of the chemical shifts of protons and carbons (essentially HC-5, C-8 and HC-12) surrounding C-7 with those of **3** allowed us to locate its hydroxy group  $\alpha$ -equatorial. The relative stereochemistry of **1** was also confirmed by the inspection of its Drieding model.

Therefore, **1** could be formulated as (2Z,4E)-5-[(1R\*,4R\*,6R\*)-1,4-dihydroxy-2,2,6-trimethylcyclohexyl]-3-methylpenta-2,4-dienoic acid. Comparing the <sup>1</sup>H and <sup>13</sup>C NMR spectra (Table 1) of **2** with those of **3**, the cyclohexyl moiety present in both compounds appeared very similar. In fact, H-7 was observed in the <sup>1</sup>H NMR spectrum as a double doublet ( $J = 9.9$  and  $5.1$  Hz) at  $\delta$  2.20 and was coupled in the COSY spectrum with H-5 and H-12. H-12, a multiplet at  $\delta$  2.06 (overlapped to the signal of Me-6), coupled with its geminal methyl group (Me-15) and with both protons of the adjacent methylene group H<sub>2</sub>C-11, which resonated, respectively, as a doublet ( $J = 7.6$  Hz), a double triplet ( $J = 12.7$  and  $4.4$  Hz), and a quintet ( $J = 7.0$  Hz) at  $\delta$  1.05, 1.91, and 1.71. The latter two protons (H<sub>2</sub>-11) coupled with the proton of the adjacent hydroxylated secondary carbon (HC-10) resonating as a quintet ( $J = 4.4$  Hz) at  $\delta$  3.77, and this latter (H-10) was also coupled with the proton of the other hydroxylated secondary carbon (HC-9), appearing as multiplet at  $\delta$  4.38. The two methyl groups (Me-13 and Me-14) gave an overlapped signal integrating for six protons at  $\delta$  1.25. The couplings observed in the HSQC spectrum allowed us to assign the signals at  $\delta$  136.7, 132.0, 114.9, 69.2, 69.0, 53.4, 38.5, 29.4, 21.4,

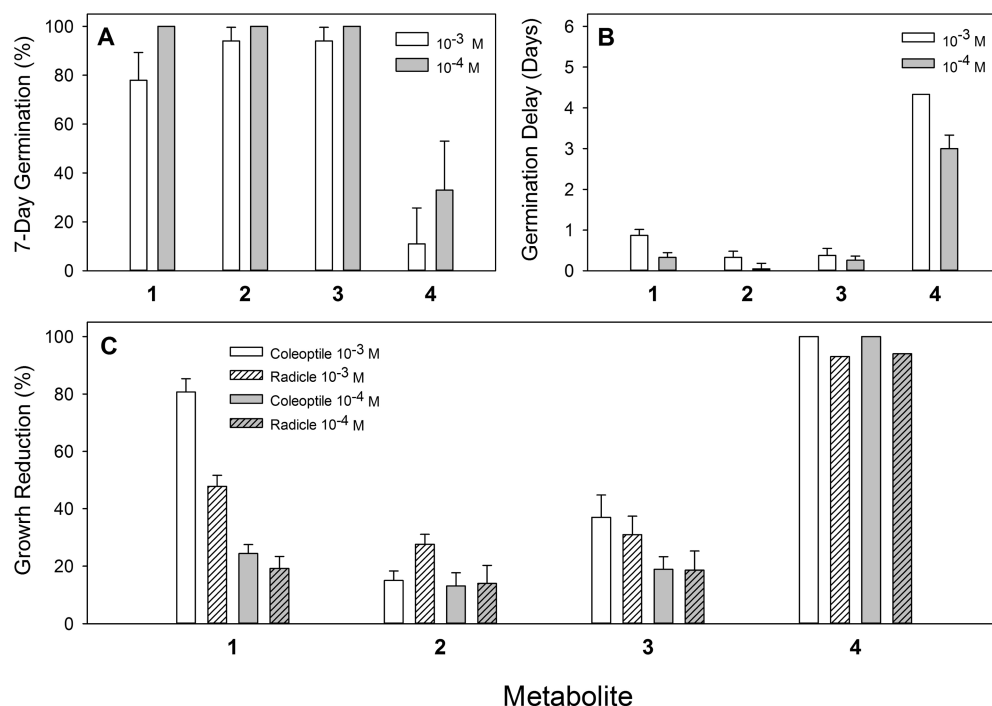
and 18.5 to C-5, C-4, C-2, C-9, C-10, C-7, C-11, Me-13 and Me-14 (overlapped signals), C-12 and Me-6 (overlapped signals), and Me-15. The couplings observed in the HMBC spectrum between C-3 with Me-6 and C-8 with Me-15 allowed the signal at  $\delta$  153.2 and 38.9 to be assigned to these two quaternary carbons. Thus, the remaining signal present at the typical chemical shift for a carboxylic group at  $\delta$  169.0 was assigned to C-1.<sup>14</sup>

On the basis of these results pyrenophoric acid **C** (**2**) could be formulated as 5-(3,4-dihydroxy-2,2,6-trimethylcyclohexyl)-3-methylpenta-2,4-dienoic acid, the same structural formula obtained for **3**. This structure was confirmed by the other couplings observed in the HMBC spectrum (Table 1) and by the data of the HRESI MS spectra. These latter recorded in positive ion mode showed the ammonium cluster  $[M + NH_4]^+$  at  $m/z$ , 286.2026, while in negative ion mode they showed the pseudomolecular ion  $[M - H]^-$  at  $m/z$  267.6075.

The only difference between **2** and **3** is in the stereochemistry of the cyclohexyl ring. H-12 was located  $\beta$ -equatorial (opposite that in **3**), and H-11a and H-11b were located  $\beta$ -axial and  $\alpha$ -equatorial as in **3**. This was deduced by measuring the values of the coupling constants in the <sup>1</sup>H NMR spectrum, between H-12 and H-7 and also between H-12 and both H<sub>2</sub>-11 and comparing these to the coupling constant values of **3**. Similarly the values recorded for the coupling of H-10 with both H<sub>2</sub>-11 and H-9 allowed us to locate this proton (H-10) as  $\beta$ -equatorial and H-9 as  $\beta$ -axial as in **3**. The correlation observed between H-9 and H-10 in the NOESY spectrum allowed us to confirm this last proton (H-9) as  $\beta$ -axial. The comparison of the chemical shifts of protons and carbons (essentially HC-5, C-8, and HC-12) surrounding C-7 with those of **3** allowed us to locate its hydrogen group  $\alpha$ -equatorial. The relative stereochemistry of **2** as depicted in Figure 1 was also confirmed by the inspection of its Drieding model.

Thus, pyrenophoric acid **C** (**2**) could be formulated as (2Z,4E)-5-[(1S\*,3S\*,4R\*,6S\*)-3,4-dihydroxy-2,2,6-trimethylcyclohexyl]-3-methylpenta-2,4-dienoic acid. Both **1** and **2**, as well as **3**, are sesquiterpenoids closely related to the well-known (+)-abscisic acid (**4**). This latter is a plant hormone with roles in environmental stress response, stomatal control of plant water balance, leaf senescence promotion, and growth inhibition. **4** also regulates other important physiological processes in plants including seed development, storage product deposition, and dormancy.<sup>16</sup> The biosynthesis and the metabolism of **4** have been extensively studied,<sup>17–19</sup> and several abscisic acid derivatives have been synthesized.<sup>20,21</sup> However, **4** is not a plant-specific compound but one found in organisms across kingdoms from bacteria to animals, suggesting that it is a ubiquitous and versatile substance that can modulate the physiological functions of various organisms.<sup>22</sup> **4** has also been previously reported as a fungal metabolite.<sup>23,24</sup> Natural abscisic acid analogues have also been isolated from fungi during recent years.<sup>8,25</sup>

The compound **1** was previously reported, in racemic mixture, as a synthetic product (named PBI-175) in a patent regarding the use of abscisic acid derivatives to enhance synchrony of germination and emergence in plants.<sup>21</sup> This racemic mixture was prepared starting from the compound ( $\pm$ )-(8R\*,10S\*)-8-acetoxymethyl-1,5-dioxaspiro[5,5]undecan-9-one, following the procedure previously reported.<sup>26</sup> Only the <sup>1</sup>H NMR and HRMS data were shown, and no results of biological activity were highlighted. In this paper **1** is described for the first time as a phytotoxic natural product isolated in its

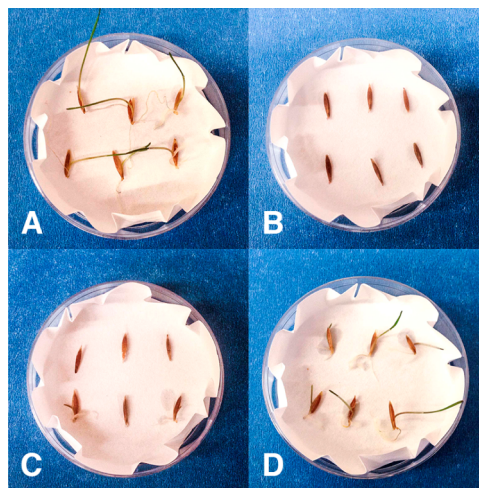


**Figure 2.** Results of host (*Bromus tectorum*) seedling bioassays with abscisic acid, 4, and the related compounds pyrenophoric acid, 3, pyrenophoric acid B, 1, and pyrenophoric acid C, 2, produced by the seed pathogen *Pyrenophora semeniperda*, at two concentrations ( $10^{-3}$  and  $10^{-4}$  M) (A) Seed germination percentage after 7 days. (B) Seed germination delay relative to the 2% DMSO control. (C) % coleoptile and radicle 5-day length reduction relative to the 2% DMSO control. Error bars represent standard error of the mean.

pure enantiomeric form from the cheatgrass seed culture of the fungus *P. semeniperda*. Other synthetic analogues related to pyrenophoric acid B were reported in the previously cited patent and in a related paper on the metabolism of biologically active abscisic acid analogues.<sup>21,27</sup>

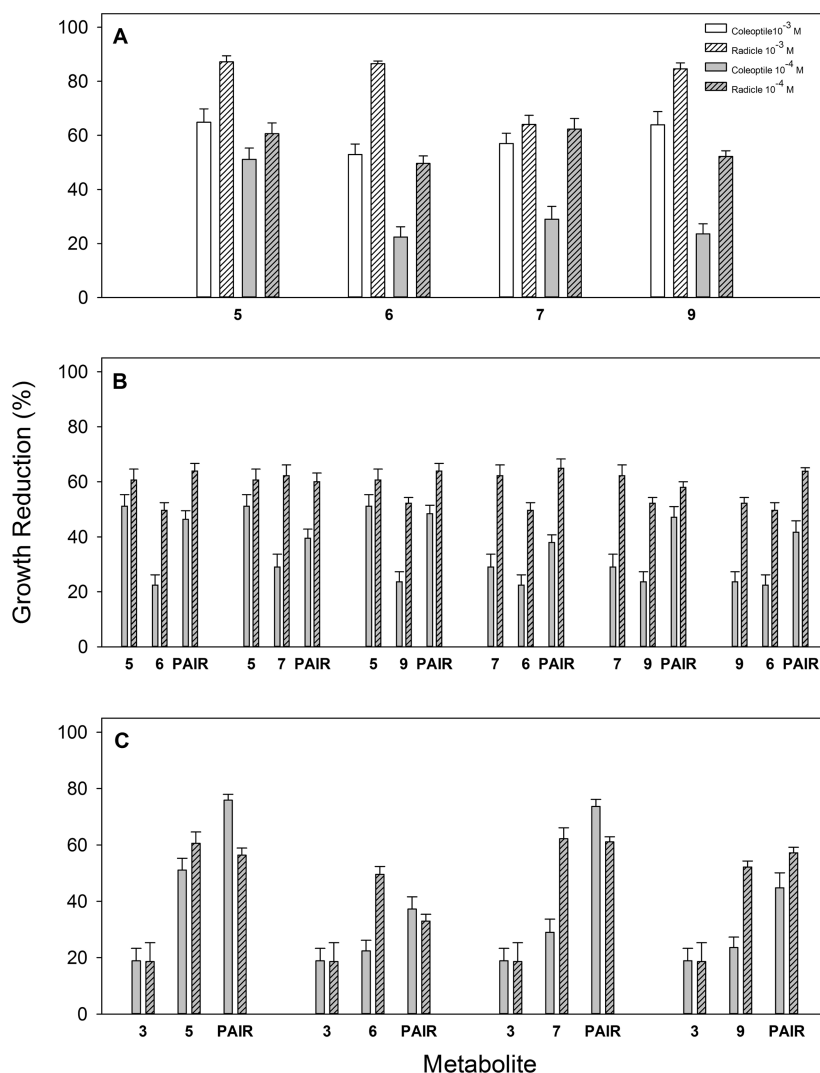
Seedling bioassays with pyrenophoric acid (3), abscisic acid (4) produced by the fungus, and the two new related compounds pyrenophoric acids B and C (1 and 2) demonstrated a hierarchical order of toxicity. All four compounds caused significant 5-day coleoptile and radicle length reductions relative to the control even at the lower concentration, demonstrating their potential role as phytotoxins (Figures 2 and 3). Not surprisingly, 4 was by far the most active compound, and was able to suppress most germination, delay the germination of seeds which did germinate, completely suppress coleoptile elongation, and greatly reduce radicle length relative to the control (Figures 2A, 2B, and 2C). We obtained a similar result in an earlier study with a synthetically produced ( $\pm$ )-abscisic acid, which completely suppressed germination of *B. tectorum* seeds.<sup>9</sup>

Among pyrenophoric acid analogs (1 and 2), only pyrenophoric acid B (1) was able to reduce 7-day germination and cause significant germination delay relative to the control (Figures 2A and 2B). It also caused significantly more coleoptile and radicle growth reduction than the other two compounds at  $10^{-3}$  M (Figure 2C). None of these effects were significant at the lower concentration. Pyrenophoric acid (3) caused more coleoptile growth suppression than pyrenophoric acid C (2), but only at the higher concentration. The relative toxicity ranking of the four compounds was therefore abscisic acid  $\gg$  pyrenophoric acid B  $>$  pyrenophoric acid  $>$  pyrenophoric acid C (4  $\gg$  1  $>$  3  $>$  2). These results allow us to consider structure–activity relationships between abscisic acid (4) and the related pyrenophoric acids (1–3). The presence at C-10 of



**Figure 3.** Host (*Bromus tectorum*) seedlings 5 days after germination for (A) the 2% DMSO control treatment, (B) abscisic acid, 4, at  $10^{-4}$  M (showing lack of germination), (C) pyrenophoric acid B, 1, at  $10^{-4}$  M, and (D) pyrenophoric acid, 3, at  $10^{-4}$  M.

the  $\alpha,\beta$ -unsaturated ketone in 4 seems to play a central role in the strong germination-inhibiting activity of this compound. The absence of this moiety in 1, 2, and 3 causes a noticeable activity reduction. The hydroxy group at C-7 in 4 and 1 also seems to be involved, as it is absent in the less active 2 and 3. Furthermore, the stereochemistry of the chiral C-7 in 4, compared with that of 1–3, could be important in increasing activity, while the difference between 2 and 3 at C-12 should be unimportant. To better understand the potential mode of action of pyrenophoric acids and which structural features are involved, suitable semisynthetic derivatives should be prepared from 1–3 and tested in seedling bioassays.



**Figure 4.** Results of host (*Bromus tectorum*) seedling bioassays showing the reduction in 5-day radicle and coleoptile length relative to the 2% DMSO control for four cytochalasins [cytochalasin A, 5, cytochalasin B, 7, cytochalasin F, 9, and deoxaphomin, 6] produced by the seed pathogen *Pyrenophora semeniperda*: (A) applied singly at two concentrations ( $10^{-3}$  and  $10^{-4}$  M), (B) applied in pairwise additive combination with each other at  $10^{-4}$  M, and (C) applied in pairwise additive combination with pyrenophoric acid, 3, at  $10^{-4}$  M. Error bars represent standard error of the mean.

In the bioassay with pure cytochalasins, all four compounds significantly reduced 5-day coleoptile and radicle length at both concentrations relative to the control, clearly demonstrating their toxicity on this host (Figure 4A). Toxicity was consistently higher at  $10^{-3}$  concentration (except for the effect of 7 on radicle growth, which was similar at the two concentrations). The growth-suppressive effect was far from additive, however, suggesting some form of saturation at higher concentrations. The negative effect on radicle growth was generally greater than the effect on coleoptile growth. There was little difference among the compounds in their impact on seedling growth at high concentration, but at low concentration, 5 caused significantly more coleoptile growth reduction than the other three compounds. In contrast with their strong toxicity to seedlings, cytochalasins had no significant effect on germination time or percentage (data not shown).

When the cytochalasins were combined additively in pairwise combinations at  $10^{-4}$  M, there was no sign of an additive effect on radicle growth suppression in five of six pairs, with suppression caused by the most toxic compound (5 or 7) not significantly less than the effect of the combined

compounds (Figure 4B). Only the combination of cytochalasin F and deoxyphomin (9 and 6) resulted in a significant radicle growth suppression increase relative to either compound alone. For coleoptile growth suppression, combinations that included 5 resulted in no significant increase in suppression relative to this compound applied singly. Combinations that included 7 but not 5 resulted in increased suppression relative to the pure compounds, while the combination of 9 and 6 resulted in a nearly additive effect. These results suggest that when more toxic cytochalasins are included in the pairwise combination, the apparent saturation effect observed at higher concentrations of pure compounds is also evident.

When cytochalasins were combined in pairwise combinations with pyrenophoric acid, there was again little indication of an additive effect on radicle growth suppression, with no significant increase in suppression beyond that achieved by cytochalasins applied as pure compounds (Figure 4C). There was actually a significant decrease in toxicity to radicles when pyrenophoric acid (3) was combined with deoxaphomin (6). In contrast to the effect on radicles, the effect of pyrenophoric acid–cytochalasin combinations on coleoptile growth suppress-



sion was at least additive or nearly so. When 7 was combined with 3, the toxicity effect on coleoptiles was clearly synergistic, with a growth reduction of 74%, or 1.54 times the reduction predicted by a strictly additive effect ( $19\% + 29\% = 48\%$ ).

In conclusion, the structures of two new sesquiterpenoids, named pyrenophoric acids B and C and isolated from *B. tectorum* culture of *P. semeniperda*, were reported. They are closely related to pyrenophoric acid, previously isolated from the same fungus, being the 7-dehydro-7-hydroxy and the 12-epimer of this acid. They are structurally related to abscisic acid, which was also isolated from the fungus in this study.

As is true for most of the secondary metabolites produced by fungi, the biological functions of phytotoxic metabolites produced by *P. semeniperda* in pathogenesis on *B. tectorum* seeds are still largely unknown. We have demonstrated here that these metabolites are produced by the fungus on seeds of the host of origin and not just in standard wheat seed culture. This makes it more likely that they have some function in pathogenesis. As cytochalasin B is produced in quantities orders of magnitude greater than other secondary metabolites known to be produced by this fungus, it seems likely that it is important in terms of pathogenesis. The detection of a series of closely related cytochalasins could suggest that they represent sequential steps in biosynthesis of the principal compound or possibly degradation products.<sup>28</sup> Cytochalasin B is well-known to interfere with cytokinesis through its action on the actin skeleton of cells, making it a logical candidate as a compound that could facilitate pathogenesis on seeds by interfering with germinant development. Similarly, abscisic acid is the most likely of the sesquiterpenoid group of compounds produced by *P. semeniperda* to play a direct role in pathogenesis on seeds because of its ability to delay or halt germination. This compound and related pyrenophoric acids were detected in very small quantities, however. The failure to detect abscisic acid and related compounds in biologically significant amounts could be an artifact of cultural conditions on autoclaved seeds. The synergistic effect of pyrenophoric acid and cytochalasin B on coleoptile growth suppression suggests that they may play complementary roles in pathogenesis, especially if the effect of cytochalasin B is saturating at relatively low concentrations, making the large disparity in production levels less important. However, this begs the question of why the fungus produces such large quantities of cytochalasin B.

An annotated genome assembly for *P. semeniperda* is now available, and the putative gene cluster for cytochalasin biosynthesis in this fungus has been identified.<sup>29</sup> Gene silencing studies to directly determine how cytochalasin B and other phytotoxins produced by *P. semeniperda* function in pathogenesis provide the most promising approach for understanding the activities in vivo of the diverse array of phytotoxic metabolites produced.

## ■ ASSOCIATED CONTENT

### ● Supporting Information

NMR and HRESI MS spectra of 1 and 2. This material is available free of charge via the Internet at <http://pubs.acs.org>.

## ■ AUTHOR INFORMATION

### Corresponding Author

\* Tel: +39 081 2539178. E-mail: [evidente@unina.it](mailto:evidente@unina.it).

## Funding

This research was funded in part by Grant JFSP-11-S-2-6 to S.M. from the Joint Fire Sciences Program of the U.S. Departments of Agriculture and Interior and in part by Programme STAR financially supported by UniNA and Compagnia di San Paolo.

## Notes

The authors declare no competing financial interest.

## ■ ACKNOWLEDGMENTS

Logistical support at Brigham Young University was provided to M.M. by Dr. Paul Savage of the Chemistry Department. A.E. is associated with Istituto di Chimica Biomolecolare del CNR, Pozzuoli, Italy.

## ■ REFERENCES

- (1) Stahlman, P. W.; Miller, S. D. Downy brome (*Bromus tectorum*) interference and economic thresholds in winter wheat (*Triticum aestivum*). *Weed Sci.* **1990**, *38*, 224–228.
- (2) Ostlie, M. H.; Howatt, K. A. Downy brome (*Bromus tectorum*) competition and control in no-till spring wheat. *Weed Technol.* **2013**, *27*, 502–508.
- (3) Brooks, M. L.; D'Antonio, C. M.; Richardson, D. M.; Grace, J. B.; Keeley, J. E.; Di Tomaso, J. M.; Hobbs, R. J.; Pellant, M.; Pyke, D. Effects of invasive alien plants on fire regimes. *Bioscience* **2004**, *54*, 677–688.
- (4) Meyer, S. E.; Clement, S.; Beckstead, J. US Patent Application Number US20130035231, 2013.
- (5) Medd, R. H. A review of the world distribution and host range of *Pyrenophora semeniperda*. *Rev. Plant Pathol.* **1992**, *71*, 891–901.
- (6) Stewart, T. E.; Allen, P. S.; Meyer, S. E. First report of *Pyrenophora semeniperda* in Turkey and Greece. *Plant Dis.* **2009**, *93*, 1351–1351.
- (7) Evidente, A.; Andolfi, A.; Vurro, M.; Zonno, M. C.; Motta, A. Cytochalasins Z1, Z2 and Z3, three 24-oxa[14]cytochalasins produced by *Pyrenophora semeniperda*. *Phytochemistry* **2002**, *60*, 45–53.
- (8) Masi, M.; Evidente, A.; Meyer, S.; Nicholson, J.; Muñoz, A. Effect of strain and cultural conditions on the production of cytochalasin B by the potential mycoherbicide *Pyrenophora semeniperda* (Pleosporaceae, Pleosporales). *Biocontrol Sci. Technol.* **2013**, *24*, 53–64.
- (9) Masi, M.; Meyer, S.; Cimmino, A.; Andolfi, A.; Evidente, A. Pyrenophoric acid, a phytotoxic sesquiterpenoid penta-2,4-dienoic acid produced by a potential mycoherbicide, *Pyrenophora semeniperda*. *J. Nat. Prod.* **2014**, *77*, 925–930.
- (10) Masi, M.; Meyer, S.; Clement, S.; Andolfi, A.; Cimmino, A.; Evidente, A. Spirostaphylotrichina W, a spirocyclic  $\gamma$ -lactam isolated from liquid culture of *Pyrenophora semeniperda*, a potential mycoherbicide for cheatgrass (*Bromus tectorum*) biocontrol. *Tetrahedron* **2014**, *70*, 1497–1501.
- (11) Berger, S.; Braun, S. *200 and More Basic NMR Experiments: a Practical Course*, 1st ed.; Wiley-VCH: Weinheim, Germany, 2004.
- (12) Kikuzaki, H.; Kayano, S.-I.; Fukutsuka, N.; Aoki, A.; Kasamatsu, K.; Yamasaki, Y.; Mitani, T.; Nakatani, N. Abscisic acid related compounds and lignans in prunes (*Prunus domestica* L.) and their oxygen radical absorbance capacity (ORAC). *J. Agric. Food Chem.* **2004**, *52*, 344–349.
- (13) Smith, T. R.; Clark, A. J.; Clarkson, G. J.; Taylor, P. C.; Marsh, A. Concise enantioselective synthesis of abscisic acid and a new analogue. *Org. Biomol. Chem.* **2006**, *22*, 4186–4192.
- (14) Breitmaier, E.; Voelter, W. *Carbon-13 NMR Spectroscopy*; VCH: Weinheim, Germany, 1987; pp 183–280.
- (15) Pretsch, E.; Bühlmann, P.; Affolter, C. *Structure Determination of Organic Compounds—Tables of Spectral Data*, 3rd ed.; Springer-Verlag: Berlin, Germany, 2000; pp 161–243.
- (16) Oritani, T.; Kiyota, H. Biosynthesis and metabolism of abscisic acid and related compounds. *Nat. Prod. Rep.* **2003**, *20*, 414–425.

- (17) Inomata, M.; Hirai, N.; Yoshida, R.; Ohigashi, H. Biosynthesis of abscisic acid by the direct pathway via ionylideneethane in a fungus, *Cercospora cruenta*. *Biosci., Biotechnol., Biochem.* **2004**, *68*, 2571–2580.
- (18) Zaharia, L. I.; Walker-Simmon, M. K.; Rodriguez, C. N.; Abrams, S. R. Chemistry of abscisic acid, abscisic acid catabolites and analogs. *J. Plant Growth Regul.* **2005**, *24*, 274–284.
- (19) Kitahata, N.; Asami, T. Chemical biology of abscisic acid. *J. Plant Res.* **2011**, *124*, 549–557.
- (20) Sondheimer, E.; Walton, D. C. Structure-activity correlations with compounds related to abscisic acid. *Plant Physiol.* **1970**, *45*, 244–248.
- (21) Abrams, S. R.; Gusta, L. V.; Reany, M. J. T.; Ewan, B. E. US Patent Application Number US005518995A, 1996.
- (22) Takezawa, D.; Komatsu, K.; Sakata, Y. ABA in bryophytes: how a universal growth regulator in life became a plant hormone? *J. Plant Res.* **2011**, *124*, 437–453.
- (23) Tudzynsky, B.; Sharon, A. In *The Mycota X: Industrial Applications*; Osiewacz, H., Ed.; Springer-Verlag: Berlin, Germany, 2002; pp 183–211.
- (24) Hartung, W. The evolution of abscisic acid (ABA) and ABA function in lower plants, fungi and lichens. *Funct. Plant Biol.* **2010**, *37*, 806–812.
- (25) Zheng, Y. B.; Lu, C. H.; Shen, Y. M. New abscisic acid-related metabolites from *Phellinus vaninii*. *J. Asian Nat. Prod. Res.* **2012**, *14*, 613–617.
- (26) Lamb, N.; Abrams, S. R. Synthesis of optically active cyclohexanone analogs of the plant hormone abscisic acid. *Can. J. Chem.* **1990**, *68*, 1151–1162.
- (27) Lamb, N.; Shaw, A. C.; Abrams, S. R.; Reaney, M. J.; Ewan, B.; Robertson, A. J.; Gusta, L. V. Oxidation of the 8'-position of a biologically active abscisic acid analogue. *Phytochemistry* **1993**, *34*, 905–917.
- (28) Scherlach, K.; Boettger, D.; Remme, N.; Hertweck, C. The chemistry and biology of cytochalasans. *Nat. Prod. Rep.* **2010**, *27*, 869–886.
- (29) Soliai, M. M.; Meyer, S. E.; Udall, J. A.; Elzinga, D. E.; Hermansen, R. A.; Bodily, P. M.; Hart, A. A.; Coleman, C. E. *De novo* genome assembly of the fungal plant pathogen *Pyrenophora semeniperda*. *PLoS One* **2014**, *9*, e87045.